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WITNESS my hand this
Ninth day of April 2003

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PROVISIONAL SPECIFICATION

Invention Title:

A composition and method for killing of tumours

The invention is described in the following statement:

A COMPOSITION AND METHOD FOR KILLING OF TUMOURS

FIELD OF THE INVENTION

The present invention relates to a method of treating solid tumours involving the use of ovine adenovirus gene transfer vectors expressing a suicide gene from a tissue specific promoter and a prodrug that can effectively destroy tumours.

BACKGROUND OF THE INVENTION

There is currently increasing interest in the use of gene directed enzyme prodrug therapy (GDEPT) in the treatment of cancer and there are several GDEPT systems are under investigation for cancer therapy. The most studied uses Herpes Simplex Virus-thymidine kinase (HSV-TK) gene transduction in combination with the pro-drug ganciclovir (GCV). HSV-TK phosphorylates GCV, converting it to a nucleoside analogue that terminates DNA synthesis, leading to the death of dividing cells. A perceived advantage of the GDEPT system is its observed local "bystander effect", namely that more cells than are transduced die due to local spread of the toxic product. This effect is in part through intercellular transport of phosphorylated GCV via gap junctions.

One of the main difficulties encountered with GDEPT is the common issue encountered with other gene therapeutics that being one of delivery.

Numerous systems have been developed for the delivery of gene therapeutics. These range from retroviruses capable of stably introducing a gene of interest to the genome of recipient cells to non-viral systems such as cationic lipids and dendrimers. In particular, gene delivery systems based on adenoviruses are currently being used with increasing frequency. These viral vectors can be produced in high yield and transfer their genetic load to a broad range of target cell types with high efficiency. While human adenoviral vectors have attracted most attention, as endemic viruses in Man their utility as gene therapy vectors is seriously compromised by high levels of pre-existing immunity against them in the human population.

SUMMARY OF THE INVENTION

The present inventors have developed a GDEPT for solid tumours based on delivery using ovine adenovirus.

Accordingly, the present invention consists in a method of treating a solid tumour of a specific tissue type in a subject, the method comprising the following steps

(i) delivering to the solid tumour a composition comprising an engineered ovine adenovirus; and

(ii) administering a prodrug to the subject,

wherein the engineered ovine adenovirus comprises a promoter active in the specific tissue and a gene encoding an enzyme which converts the prodrug to a cytotoxic metabolite, the gene being under the control of the tissue specific promoter.

In a preferred embodiment the specific tissue type is prostate tissue. In this embodiment it is preferred that the tissue specific promoter is the prostate specific membrane antigen promoter or the probasin promoter. It is also preferred that the ovine adenovirus further comprises a transcriptional enhancer element, preferably the enhancer element from the prostate specific membrane antigen gene.

The prodrug/enzyme may be a[illegible]ber of combinations known in the field. Examples of such enzyme[illegible]apies include thymidine kinase with ganciclovir, thymidine kina[illegible]acyclovir, bacterial cytosine deaminase with 5-flurocytosine, human cytochrome P450 with cyclophosphamide or ifosfamide, thymidine phosphorylase with 5'-deoxy-5-flurouridine, cytosine kinase with cytosine arabinoside, *E. coli* GPT with 6-thioxanthine, *E. coli* nitroreductase with 5(-aziridine-1-yl)-2,4-dinitrobenzamide, and bacterial purine nucleoside phosphorylase coupled with 6-methylpurine-2-deoxyriboside or fludarabine.

It is presently preferred, however, that the enzyme is a purine nucleoside phosphorylase (PNP), and the prodrug is a purine pro-drug which is converted by PNP to a toxic purine metabolite. The currently preferred prodrugs are 6-methyl purine-2-deoxyriboside (6MPDR) and fludarabine; the toxic products produced by PNP on these substrates are 6-methyl purine (6MP) and 2-fluoroadenine (2FA) respectively.

Fludarabine is approved for clinical use but its Km for PNP is a ~1000-higher than that for 6MPDR. However, its PNP metabolite, 2FA is ~100-fold more potent as a growth inhibitor than 6MP, the metabolite of 6MPDR. Through their incorporation into RNA as well as DNA the metabolites produced by this enzyme/prodrug system kill non-dividing as well as dividing cells.

This enzyme/prodrug system also induces a more efficient local "bystander effect" than the HSV-TK/GCV system because the metabolites are non-phosphorylated purines that can diffuse freely across the membranes of non-transduced cells.

The ovine adenovirus (OAdV, formerly designated OAV) is preferably the ovine adenovirus described in PCT/AU95/00453 and/or PCT/AU96/00518, the disclosures of which are hereby incorporated by cross-reference. The sequence of this virus is provided in GenBank Accession No. U40839. Reference to sequence Nos. is made in relation to this deposited sequence.

The cloning of a full length, infectious OAdV287 genome into the plasmid vector pBluescribe has been described (Vrati S, Macavoy ES, Xu ZZ, Smole C, Boyle DB and Both GW (1996) "Construction and transfection of ovine adenovirus genomic clones to rescue modified viruses", Virology **220**: 200-203). This primary recombinant product, pOAdV100, allows for the convenient release of the linearised, infectious OAdV287 genome by digestion with the restriction endonuclaese KpnI. Further modifications to pOAdV100 have been made to allow convenient insertion of foreign DNA into the viral genome without disrupting essential virus replication and packaging functions. One such modified plasmid is pOAdV600. To create pOAdV600 the ~7.1 kb SphI/SalI fragment of pOAdV100 was subcloned into pALTER-1(Promega Corp., Madison, WI). Using a mutagenesis kit (Altered sitesII, Promega Corp.), unique ApaI and NotI sites were inserted using a synthetic oligonucleotide (5' . . . . .GGG CCC TGA ATC TGC GGCCGC . . . . . .3') where the flanking sequences (indicated by the dots) were designed to allowed insertion of the oligonucleotide sequence indicated immediately 5' to base 26,676 of the OAdV287 sequence. The modified SphI/SalI fragment was then recloned into SphI/SalI cut POAdV100 to produce pOAdV600. In a related fashion, the same sequence indicated above was inserted between nucleotides 22129 and 22130 of the OAdV287 genomic sequence resulting in the production of pOAdV200. Both pOAdV200 and pOAdV600 have been used to prepare recombinant ovine adenoviruses carrying a variety of gene expression cassettes including cassettes useful for a PNP-based GDEPT for the treatment of cancers.

By varying the promoter used to drive expression of the enzyme component of a GDEPT, viruses can be tailored either for utility in the

treatment of a wide range of tumours or to provide targetted tumour type and/or tissue specificity.

OAdV220 provides an example of a therapeutic virus that could be used in the treatment of a wide variety of tumours. In this virus transcription of the PNP gene from E. coli is driven by from the constitutive RSV promoter (long terminal repeat from the Rous Sarcoma Virus) and terminated by the polyadenylation sequence from the bovine growth hormone gene. This expression cassette is inserted into ApaI/Not1 polylinkerof pOAdV200 (ie is in site 1 of the OAdV287 genome). Use of a strong constitutive promoter such as that found in the RSV long terminal repeat allows for high levels of PNP expression in a wide variety of cell types and tissues.

On the other hand, to maximise the local effect of GDEPT while minimising the potential for non-specific toxicity (eg. through virus uptake in the liver or other tissues surrounding the injection site) it is desirable to restrict expression of the pro-drug activating enzyme to target tissues. For any particular cancer type this can be achieved by placing the GDEPT gene expression cassette under the control of a promoter that is active only in the tissue type from which the cancer is derived. In the case of prostate cancer a candidate for such a gene regulator is the promoter for prostate specific membrane antigen.

Prostate specific membrane antigen (PSMA) is a protein with folate hydrolase activity. It was first identified as an antigen present on the membrane of a prostate cancer cell line as well as normal and cancerous prostate epithelium. Its expression is largely restricted to the prostate. Lower levels of expression have been detected in the brain, the small intestine and in a subset of kidney tubule cells (reviewed in (Fair *et al.*, 1997 Prostate-specific membrane antigen. *Prostate* **32**(2), 140-148). More recently it was established that PSMA is also expressed in the neovasculature of a wide range of tumour types, but not in normal vasculature (Chang *et al.*, 1999 Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res* **59**(13), 3192-3198; Liu *et al.* 1997 Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. *Cancer Res* **57**(17), 3629-3634; Silver *et al.*, 1997 Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin Cancer Res* **3**, 81-85). Both cDNA and genomic clones of PSMA have been isolated and characterised

(Israeli *et al.*, 1993. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res* **53**, 227-230; O'Keefe *et al.*, 1998 Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochimica Et Biophysica Acta* 1443: 113-27).

A transcriptional enhancer sequence (PSME) has been isolated from the PSMA gene and demonstrated in a series of transfection studies in multiple cell types to confer high level enhancement of transcription that is restricted to PSMA-expressing prostate cells. PSME has been shown to activate transcription from both its own promoter and those of heterologous genes. Further information regarding this enhancer element may be found in PCT/AU00/01143, the disclosure of which is incorporated herein by cross-reference.

The 430 bp proximal promoter from the rat probasin gene has also been shown to direct expression specifically to prostate cells both *in vitro* and in transgenic mice (Brookes *et al.*, 1998 Relative activity and specificity of promoters from prostate-expressed genes. *Prostate* **35**(1), 18-26; Greenberg *et al.*, 1994 The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specific to the prostate in transgenic mice. *Mol Endocrinol* **8**(2), 230-239; Kasper *et al.*, 1994 Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene. *J Biol Chem* **269**(50), 31763-9). A chimaeric promoter consisting of PSME and the probasin proximal promoter, when linked to a reporter gene, produced expression levels of the reporter product that were about ten times higher than those obtained with PSME linked to its own, PSMA, promoter. Importantly the tissue specificity of expression was also maintained.

In OAdV623, transcription of the PNP gene is under the control of a the chimaeric promoter described above, ie. an ~ 1kb fragment including bases 14760 -15804 of the human prostate specific membrane antigen gene sequence (O'Keefe *et al.*, 1998 Mapping, genomic organisation and promoter analysis of the human prostate-specific membrane antigen gene. *Biochimica Et Biophysica Acta* **1443**(1-2), 113-27).operatively coupled to the 430 bp proximal promoter from the rat probasin gene. Transcription of this expression cassette is terminated with the bovine growth hormone poly adenylation sequence (BGH Poly A). This expression cassette is inserted into site III of the OAdV287

sequence. OAdV623, therefore, provides an example of a therapeutic virus that could find greatest utility in the delivery of a GDEPT for the treatment of prostate cancer.

In a further preferred embodiment the composition comprising the engineered ovine adenovirus further comprises a lipid. It is presently preferred that the lipid is a cationic lipid. Preferred cationic lipids are those based on a TRIS linkage. Exemplary compounds are described in US patents 5,854,224 and 5,906,922, the disclosures of which are incorporated herein by cross-reference. Particularly preferred cationic lipids are shown in Figure 9.

It is preferred that the composition comprising the engineered ovine adenovirus and cationic lipid is delivered directly to the solid tumour by injecting the composition into the tumour.

As used herein the term "treating" is used in its broadest sense and is intended to encompass treatment which results in eradication of the tumour, causes a decrease in tumour size or causes a decrease in rate of tumour growth.

Cationic lipids have been shown to [illegible] transfer of DNA into cells and to enhance the infectivity of a rang[illegible] addition, lipids are also known to enhance immune responses wher[illegible]exed with specific antigens. Lipids and lipopeptides, when formulated with specific peptides or antigenic agents have been shown to potentiate the immune responses to these agents. Vaccination of this sort leads to production of both Th1 and Th2 immune responses enhancing the immune response raised against weakly immunogenic antigens.

It has also been shown that immune responses against specific tumours can be elicited by vaccination of tumour-bearing animals with antigens specific for that tumour (often prepared by recombinant DNA approaches) formulated with cationic lipids. Such vaccination suppressed both primary and metastatic tumour growth.

In summary, the present inventors have shown that cationic lipids can also enhance the ability of ovine adenovirus gene transfer vectors to infect a range of cell types including prostate cancer cells. The inventors have developed ovine adenovirus vectors that carry the *E. coli* gene encoding purine nucleoside phosphorylase operably linked to either the highly active, constitutive promoter of the Rous sarcoma virus 3' long terminal repeat or the prostate-specific, chimaeric PSM enhancer/probasin promoter and the bovine

growth hormone poly adenylation sequence. Schematic representations of these recombinant viral genomes are shown in Figure 1. The inventors have demonstrated that injection of these viruses complexed with cationic lipid into tumours carried in mice and derived from prostate cancer cells coupled with treatment with the prodrug fludarabine leads to a significant reduction in tumour growth over that observed with virus alone. Surprisingly, this enhanced tumour killing was not mediated solely by the increased efficiency of viral transduction of the tumour. Rather the presence of lipid also markedly enhanced the infiltration of the tumour mass by tumour infiltrating leucocytes. Immunohistochemical analysis revealed that these infiltrating lymphocytes were of subtypes commonly associated with anti-tumour immune responses. Thus a lipid, when applied to a primary tumour in conjunction with a virus-borne GDEPT system, leads to an unexpected promotion of an immune response against the tumour.

DETAILED DESCRIPTION

In order that the nature of the current invention may be more fully understood preferred embodiments thereof will now be described with reference to the following non-limiting examples.

Example 1. OAdV Carries Transgenes Genes into Tumours in vivo.

To determine whether OAdV could deliver genes into tumours *in vivo*, cells from the human prostate cancer, androgen independent cell line PC3 (1.5×10^6 cells/tumour) were implanted subcutaneously (sc) in BALB/c (nu/nu) ["nude'] mice. Tumours were allowed to develop for 3-6 weeks until they had grown to around 5 mm in diameter. Tumours were then injected with 1.2×10^8 plaque forming units (pfu) of OAdV216, an ovine adenoviral vector expressing the human placental alkaline phosphatase gene. Tumours were harvested four days post injection, frozen, sectioned and stained for reporter gene activity. Figure 2 shows extensive alkaline phosphatase staining of a representative tumour section indicating that wide dissemination of the virus within the tumour and infection of tumour cells occurred.

Example 2. OAdV Delivers PNP Activity into Tumours *in vivo*.

RM-1 is an androgen-independent cell line derived by transformation of cells from the genital ridge of embryonic C57BL/6 mice with ras and myc oncogenes. When implanted into C57BL/6 mice in the appropriate locations these cells can from tumours either subcutaneously or in the prostate. Lung pseudometastases can also be formed when cells are injected intravenously (iv) via the tail vein (Hall *et al.*, 1997 Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. *Int J Cancer* 70, 183-187; Hall et al.1998 Induction of potent antitumor natural killer cell activity by herpes simplex virus thymidine kinase and ganciclovir therapy in an orthotopic mouse model of prostate cancer. *Cancer Res* 58: 3221-3225).

To determine whether, in an immunocompetent animal, a single intratumoural administration of a PNP-expressing OAdV virus could lead to PNP expression that would be sustained over a period of prodrug treatment that might be used for therapy, subcutaneous RM-1 tumours were established in C57BL/6 mice. Tumours were initiated by injection of 2.5 x 10^5 RM-1 cells into the flanks of C57BL/6 mice. On day 0 when tumours had reached 5 mm diameter they were injected with 20 µL of a preparation of OAdV220 (Figure 1) containing 6 x 10^9 virus particles (VP) of virus in virus storage buffer. Tumours were excised 1-6 days after treatment and homogenised in 400 µl of ice cold homogenisation buffer (50 mM potassium phosphate buffer, pH 7.4). Homogenates were transferred to clean eppendorf tubes, snap frozen on dry ice then thawed in a 37°C water bath. Tubes were mixed again and subjected to two further rounds of freeze thaw treatment to complete cell lysis. Cell debris was removed by centrifugation for 5 minutes at 15,000 x g. Supernatants were removed to new sterile microfuge tubes and placed on ice. Aliquots from each sample were removed for estimation of protein content using a Pierce BCA protein estimation kit. Volumes of homogenate corresponding to 500 µg of soluble protein were removed to fresh tubes for quantification of PNP activity.

To assay for PNP activity, phosphate buffer (described above) was added to the homogenate to a final volume of 1.1 mL. To each sample was added 100 µL of phosphate buffer containing 500 nmols of fludarabine (substrate for the PNP assay). Contents were gently mixed then tubes were incubated at 37°C for 16h. The reaction was stopped by heating the samples to 100°C for 5 min. Tubes were then centrifuged at 15,000 x g for 5 min to remove any remaining

debris. The amount of fludarabine converted to 2FA by the PNP present in the tumour lysates was determined by analytical high performance liquid chromatography.

For substrate/product separation a Millipore C18 column was equilibrated with 50 mM $NH_4H_2PO_4$/5% Acetonitrile for 30 min (flow rate 1 mL/min). Sample (50 µL) was applied to the column and fractionated in the same ammonium phosphate/acetonitrile buffer (isocratic gradient) at a flow rate of 1.5 ml/min. The elution profile was detected by UV absorbance at 254 nm and the relative amounts of material in the substrate and product peaks were determined by calculating the area under the curve for each peak. These HPLC analyses showed that, under these conditions, the different lysates converted between 5 and 35% of available substrate to 2FA (Figure 3). The results confirm that OAdV220 can effectively deliver the PNP gene expression cassette into tumours in vivo in an immunocompetent host and that PNP expression can be sustained over at least a six day period.

Example 3. OAdV Delivers Genes to Primary Human Prostate Tissue.

As a bridge between our studies in the animal models and clinical [illegible] we have examined whether our viral vectors can deliver reporter genes to post-operative human prostate tissues. Tissue slices, obtained from either transurethral resections of the prostate (TURP) or from radical prostatectomy specimens from patients undergoing surgery for various conditions of the prostate, were cut into small fragments. These fragments of prostate tissue were placed on matrigel on pieces of gel foam in tissue culture wells and culture medium (T-medium (Thalmann GN, Sikes RA, Chang F-M, Johnston DA, von Eschenbach AC and Chung LWK "Suramin-introduced decrease in prostate specific antigen expression with no effect on tumour growth in the LNCaP model of human prostate cancer" J. Nat. Cancer Inst. 88: 794-801) was placed in the wells to the level of the gel foam, but not covering the tissue. After overnight incubation the tissues were transduced with OAdV217A, a virus carrying the Green Fluorescent Protein (GFP) reporter gene under the control of the cytomegalovirus (CMV) immediate early promoter. Green fluorescence of the tissue provided evidence for virus transduction. Viability of the tissue was assessed by propidium iodide exclusion. While this is not a quantitative assay, the representative result shown in Figure 4 and other data not shown

demonstrated that, where the tissue was viable, the OAdV vector successfully transduced the primary human prostatic tissue. Samples successfully transduced ranged from benign hyperplastic to high-grade tumour tissue.

Example 4. Ovine adenovirus-borne, PNP-based GDEPT suppresses growth of human prostate cancers in nude mice.

The ability of the PNP-based GDEPT system to suppress tumour growth in vivo was tested using subcutaneous human PC3 tumours grown in nude mice. Once the tumours were established (see example1), on day 0 two groups of mice received one intratumoural injection of 1×10^{10} VP of OAdV220. Two further groups received virus storage buffer alone. Following this treatment all animals in one of the virus groups and all animals in one of the no-virus groups received a daily intraperitoneal (ip) injection of fludarabine (75 mg/m2/day) for the next five days. Animal weights and tumour volumes were recorded twice weekly. The vector alone group was compared to the nil virus, nil prodrug group. The group receiving vector with fludarabine was compared with the group that received fludarabine alone. The OAdV220 vector alone caused a 30% reduction in tumour growth. However, tumour growth suppression was increased to 71% when virus in conjunction with Fludarabine.

Figure 5 shows the data presented as the number of responding tumours at day 4 (just after the start of treatment), day 25 (whilst control mice are still alive) and day 53, after all control mice had died. The cut off for response was arbitrarily taken as a tumour volume of 600 mm^3, half the maximum tumour volume (1200 mm^3) allowed before mice had to be culled from tumour burden. By day 25, 13 of 14 tumours treated with OAdV220 plus fludarabine were in the responder category compared with 6 of 9 which received fludarabine only, and 9 of 12 which received virus only. By day 53, corresponding figures were 7/8 responders (OAdV plus Fludarabine), 1/6 (OAdV220 only) and 0/5 (Fludarabine only). By day 58 post treatment, 57% of mice receiving OAdV220 plus Fludarabine were alive compared with 14% receiving vector alone.

Example 5. Ovine adenovirus-borne, PNP-based GDEPT suppresses growth of murine tumours in immunocompetent mice.

To confirm that the GDEPT treatment could suppress tumour growth in the presence of a functioning immune system, C57BL/6 mice were inoculated

subcutaneously with 2.5 x 10^5 RM-1 tumour cells on day −5 to establish tumour growth. On day 0, these tumours were injected with OAdV220 at 6 x 10^9 VP/tumour. On days 1-5 mice received either 600 mg/m2/day of Fludarabine or an equivalent volume of saline by intraperitoneal administration. Animal weights and tumour volumes were recorded twice weekly. Figure 6 shows that OAdV plus Fludarabine reduced overall tumour growth by 35% when compared to controls. At day 12, when group comparison was still possible (i.e., control mice were still alive), there was a 59% reduction in mean tumour volume in the OAdV220/fludarabine group compared with fludarabine alone. Figure 7 shows that 30% of OAdV220/Fludarabine treated mice survived for an extra 3 days compared with control mice which all died by day 16. These results are particularly encouraging given the aggressiveness of this particular tumour model.

Example 6. Increasing transduction levels of RM-1 tumour cells enhances the antitumour effect of an OAdV-borne GEDEPT on subcutaneous prostate tumours in immunocompetent mice.

RM-1 tumour cells are a very aggressive cancer model. Thus the effect shown in example 5 are likely to under estimate the efficacy that might be expected in the treatment of human disease. It was thought that a better model for treatment of human disease, where prostate cancers grow more slowly, might be achieved if the tumours could be transduced with virus earlier than 5 days post inoculation. In practice, however, it is too difficult to accurately inject tumours earlier than day 5 in this model. An alternative approach was therefore adopted in which RM-1 tumour cells were transduced with virus *in vitro* prior to their inoculation into mice.

RM-1 cells 3 x 10^7 were transduced in culture with 1 x 10^3 or 1 x 10^2 particles/cell of OAdV220 or mock transduced with virus dilution buffer on day -1. On day 0, 12 mice/group were subcutaneously injected with 2.5 x 10^5 virus- or mock-transduced cells. Fludarabine was then administered from days 1-5 at 600 mg/m2/day. Tumour volume and animal survival were monitored three times per week. A line of best fit was generated for each treatment curve and the ability to suppress tumour growth or increase animal survival was determined from the days taken to reach the midpoint of tumour growth or animal survival respectively.

Figure 8A traces the increase in the mean tumour volume with time after implantation. The GDEPT effect showed a clear dose response related to the virus input/cell. At day 15 post treatment, cells transduced with 1 x 10^3 particles per cell plus Fludarabine showed a 90% reduction in mean tumour volume compared with mock transduced cells that also received Fludarabine. In contrast cells transduced with 1 x 10^2 particles/cell and receiving Fludarabine had only a 41% reduction in mean tumour volume compared with Fludarabine treated, mock transduced cells. Fludarabine alone caused a 17% reduction in mean tumour volume compared with nil treatment at day 15. Overall, when cells were pre-transduced with 1 x 10^3 particles per cell and receptor animals treated with Fludarabine, a 74% reduction in tumour growth was observed when compared with tumours derived from mock-transduced cells where the host animals also received Fludarabine. Figure 8B shows that survival was markedly increased by GDEPT treatment. 80% of mice receiving cells transduced with 1 x 10^3 virus particles per cell in combination with the pro-drug were still alive at day 34 whereas all control mice had died by day 28. There was no improved survival of animals receiving RM-1 cells treated with 1 x [illegible] particles per cell and Fludarabine and the matched minus Fludarabine control. These results suggest a dose responsiveness to the viral input, which presumably reflects a dose responsiveness to the amount of PNP product.

Example 7. Cationic lipids enhance virus transduction of prostate cancer cells both in vitro and in vivo.

To investigate whether cationic lipids could be used to enhance viral transduction of human prostate cancer cells, two human prostate cancer cell lines, PC3 and LNCaP, were cultured in 96 well plates and treated with either a human or ovine adenovirus carrying a reporter gene, ± cationic lipid and the relative amounts of reporter gene expression determined. PC3 cells were cultured in RPMI medium + 10% FCS. LNCaP cells were cultured as previously described (Thalmann GN et al 1996 "Suramin-induced decrease in prostate-specific antigen expression with no effect on tumour growth in the LNCaP model of human prostate cancer". J Nat. Cancer Inst 88: 794-801). On day −1 cells were split into the wells of a 96 well culture tray (2 x 10^4 cells per well for both cell lines; for LNCaP cells, wells of the culture trays were pre-treated with 10% fibronectin for 30 min prior to addition of cells to enhance the adherence of the cells to the tray during cell culture and the various washing steps

involved in the enzymatic assays). On day 0 four identical serial dilutions of virus in serum free medium were prepared across the columns of a microtitre plate (50, 25, 12.5 and 6.25 x 10^8 VP per 100 µl). In another microtitre tray four identical serial dilutions of Tris conjugated cationic lipid (CS60 or CS87 see Figure 9), were prepared down the rows of the plate (21, 10.5, 5.25 and 0 µM, volume 100 µL). Virus dilutions (100 µL) were transferred to the corresponding wells in the lipid tray. The mixture was allowed to stand at room temperature for 15 min before application to cells. Cultures were returned to the CO_2 incubator for four hours to allow viral transduction. Foetal Calf Serum was then added to a final concentration of 10% and the plates returned to the incubator. Two days after viral infection the viability of the cultures was examined using the MTS assay. Wells were then washed twice with PBS, cell lysis buffer (50 µL, 10mM Tris, 0.2% Triton X100, pH 8.0) added to each well and plates frozen at –70°C.

The expression cassette in the human Ad5 vector contained the E. coli β-galactosidase gene under the control of the RSV promoter. Assay for transgene expression was essentially as described in Felgner JH et al. 1994 (Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 269:2550-61). 100 µL of substrate buffer (1 mg chlorophenol red galactopyranoside in 60 mM sodium phosphate buffer pH 8.0, 1 mM magnesium sulphate, 10 mM potassium chloride, 50 mM betamercaptoethanol) was added to each well and colour allowed to develop at room temperature. Betagalactosidase activity was quantified relative to a standard curve prepared from purified enzyme

The expression cassette in the OAdV vector carried the human placental alkaline phosphatase reporter gene. Transduction, viability assays and cell lysis steps were all performed as described above. Upon thawing, the wells were covered with parafilm and plates incubated for 30 min at 65°C. Samples were cooled on ice then 25 µL of each lysate was transferred the wells of a clean, black microtitre plate. Human placental alkaline phosphatase activity was quantified using the Roche Molecular Biochemicals "AttoPhos substrate set", Cat# 1681 982 by reading fluorescence from each sample against those obtained from a standard curve prepared from purified enzyme.

Formulation of both human and ovine Ads with Tris-conjugated cationic lipids significantly enhanced viral transduction of human prostate cancer cells

in culture. Typically enhancements of around 15 fold and 9-18 fold are observed for the human and ovine vectors respectively (Figure 10).

Lipid also enhanced viral transduction of tumours growing in vivo. Tumours derived from the human prostate cancer cell line LN3 were established in male nude mice by subcutaneous injection of 2 x 10^6 cells diluted 1:1 with matrigel (100 µL) and allowed to grow to a size of 5 x 5 mm. Tumours were injected with 10^{10} VP of OAdV623 (a vector carrying the *E. coli* purine nucleoside phosphorylase (PNP gene) under the control of the PSM/Pb promoter (Figure 1)) in the presence or absence of cationic lipid CS87 (4 tumours per group). Tumours were excised 3 days later and homogenised as described in Example 2. Samples of homogenates containing 500 µg of soluble protein (1.1 mL) were incubated with 100 µl of 5 mM 6MPDR for 2 h at 37°C. The amount of 6MPDR converted to 6MP by the PNP present in the tumour lysates was determined by analytical high performance liquid chromatography under conditions identical to those described in Example 2. Figure 11 shows absolute percentage conversion values calculated for each tumour plus mean values and associated standard errors for all tumours in the different treatments. These experiments revealed that, although we tested both the number of injected tumours expressing PNP and the level of transgene expression was higher in tumours treated with the lipid/virus formulation.

Example 8. Effect of GDEPT ± lipid on intraprostatic RM-1 tumours.

In order to mimic clinical prostate cancer in an immunocompetent animal model, on day 1 RM-1 tumours were established in the prostate of C57BL/6 mice by intraprostatic injection of 5 x 10^3 RM-1 cells in ~ 50 µL per mouse. On day 4, these mice received an intraprostatic injection of 1 x 10^{10} VP/tumour of OAdV220, either alone or formulated with 10 µM CS87 (see groups below). Fludarabine was then administered ip at 600 mg/m2/day to appropriate groups from days 5-10. On day 6, pseudometastases were induced in the lungs by intravenous injection of 2.5 x 10^5 RM-1 cells in ~ 50 µL per mouse. Because only 30 intraprostate injections can be performed per day, the experiment was set up on 4 different days, although care was taken not to upset the timing of the experiment. The mice were weighed twice weekly, and sacrificed on day 18. Tissues were harvested for analysis (prostate volume, prostate weights and assessment of immune cell infiltrate by histology).

Treatment groups were as follows:

Group A OAdV220 alone ± fludarabine
Group B Nil treatment (storage buffer) ± fludarabine
Group C OAdV220 plus 10 μM CS87 ± fludarabine
Group D 10 μM CS87 alone ± fludarabine

Analysis of tumour volumes in the prostate revealed considerable variation between mice, possibly because the architecture of the organ was disrupted when the intraprostate virus injections were given. Moreover, there were some differences between the results obtained on different days, due to experimental variability. However, within each group (plus or minus Fludarabine in one situation per day) the results were consistent. The results of this study, shown in Figure 12, indicate that Fludarabine alone caused some repression of tumour growth (17% decrease in tumour weight and 12% decrease in tumour volume when compared with tumours receiving no treatment. Similarly OAdV220 plus fludarabine, or CS87 plus fludarabine caused 27% and 30% reduction in tumour [illegible] and 18% and 23% reduction in tumour weights respectively wh[illegible] with the appropriate virus or lipid alone controls. The most marked [illegible] on tumour growth was seen with a combination of OAdV220 plus CS87 plus fludarabine. This combined therapy decreased tumour volume by 58% ($p<0.05$, *) and tumour weight by 53% ($p<0.05$, *).

Example 9. OAdV viruses carrying the PNP gene under the control of the chimaeric PSM/PB promoter displays prostate specific PNP expression.

A chimaeric promoter carrying the 1kb PSM enhancer coupled to the 430 bp proximal promoter from the rat probasin gene (PSM/PB) was linked to the PNP gene/bovine growth hormone polyadenylation sequences. This expression cassette was incorporated into the OAdV genome at two different insertion sites (site 1 and site 3), producing viruses OAdV223 and OAdV623 respectively (see Figure 1). Different cell types were infected with OAdV223, OAdV623 or viruses in which the PNP gene was under the control of the strong constitutive RSV or CMV promoters (OAdV220 and OAdV222 respectively). Two days after infection with the appropriate virus, cell extracts were prepared and PNP activity measured as described in example 2 but using 6MPDR as a substrate.

Figure 14 shows that, in the context of the viral genome and OAdV infection, the PSM/PB promoter retains a high degree of tissue specificity. OAdV623 produced a greater level of gene expression than OAdV223 in the human prostate cancer cell types. In LNCaP and LN3 prostate cancer cells expression from the PSM/Pb element produced considerably more (4–8 fold) PNP activity than the RSV promoter but, significantly, in non-prostate cell lines these relative activities were was reversed. In these later cell types expression favoured the RSV promoter, about 4 and 5 fold for MCF-7 and HEK293 cells, 8 fold for HepG2 cells and 32 fold for MRC-5 cells) (Figure 14). Using RSV promoter activity to normalise the data, the nett effect is a preferential level of PSM/Pb promoter activity in LN3 and LNCaP cells of 15-40 fold over MCF-7 and HEK293 cells, 32-64 fold over HepG2 cells and 128-256 fold over MRC5 cells, respectively.

Example 10. An OAdV-delivered, prostate-specific GDEPT is active against human prostate tumours grown in Nude Mice

V623 contains an expression cassette carrying the PNP gene under [illegible] of the prostate-specific promoter, PSM/Pb. This promoter is only active in human prostate cells. To test whether this virus in combination with lipid and prodrug could elicit an antitumour response against human prostate cancer, tumours derived from the human prostate cancer cell line LN3 were established in nude mice and treated as described below.

LN3 tumours were established in male nude mice by subcutaneous injection of 2 x 10^6 cells diluted 1:1 with matrigel (100 µL) and allowed to grow to a 5 x 5 mm size. Ten tumour bearing mice per group, each carrying 2 tumours, were injected intratumorally with OAdV623 at 2 x 10^{10} VP/tumour ± 10 uM CS87 on day 0. Animals then received fludarabine intraperitoneally at 75 mg/m²/day on days 1 to 5. Animal weights and tumour volumes were recorded twice weekly. The vector alone groups were compared to the nil virus, nil prodrug group. Groups receiving vector with fludarabine were compared with the group that received fludarabine alone. A line of best fit was generated for each treatment curve. The ability to suppress tumour growth or increase animal survival was determined from the days taken to reach the midpoint of tumour growth or animal survival.

The effects of treatment on tumour growth are shown in Figure 15A. OAdV623 alone caused marked suppression of tumour growth, overall 55%,

compared with treatment with buffers alone ($p>0.05$, NS). Fludarabine also inhibited LN3 tumour growth when compared with the nil virus, nil prodrug group (58%) ($p>0.05$, NS). The combination of OAdV623 plus fludarabine caused a further decrease in tumour growth over that resulting from fludarabine alone (31%). This effect was even greater when lipid was also used with the combined treatment (OAdV623 plus CS87 plus fludarabine) resulting in a 64% greater suppression of tumour growth than fludarabine alone. In the absence of virus, lipid did not enhance the tumour suppressive effect of fludarabine alone. Compared with controls which received buffer only, OAdV623 plus fludarabine resulted in a 71% reduction in overall mean tumour volume ($p>0.05$, NS), whilst OAdV623 plus CS87 plus fludarabine significantly reduced LN3 tumour growth by 85% ($p<0.05$, *).

Figure 15B shows that the combinations of OAdV623 with fludarabine treatment, both in the presence and absence of lipid, substantially extend the life of the tumour bearing animals.

A gene directed enzyme prodrug system based on PNP may have certain advantages for the treatment of slow growing cancers such as prostate cancer. However it will be recognised by those skilled in the art that any enzyme prodrug combinations could be used in this anti-tumour therapy. Furthermore it will be recognised that different enzyme prodrug combinations may have specific advantages for the treatment of various other tumour types. While the examples provided relate to the treatment of prostate cancer, the principle of using an OAdV vector carrying a gene for an enzyme prodrug combination either under tissue specific or constitutive regulation, either formulated with a cationic lipid (where that lipid need not be limited to a Tris conjugate lipid) or unformulated, when applied directly to a tumour with the prodrug delivered systemically, is applicable to the treatment of any solid tumour to which the therapeutic can be delivered. It will also be appreciated that the presence of a lipid in the tumour tissue during the tumour killing process would further enhance the killing of this broader suite of solid tumours.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 28th day of March 2002

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION
Patent Attorneys for the Applicants:

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PATENT SERVICES

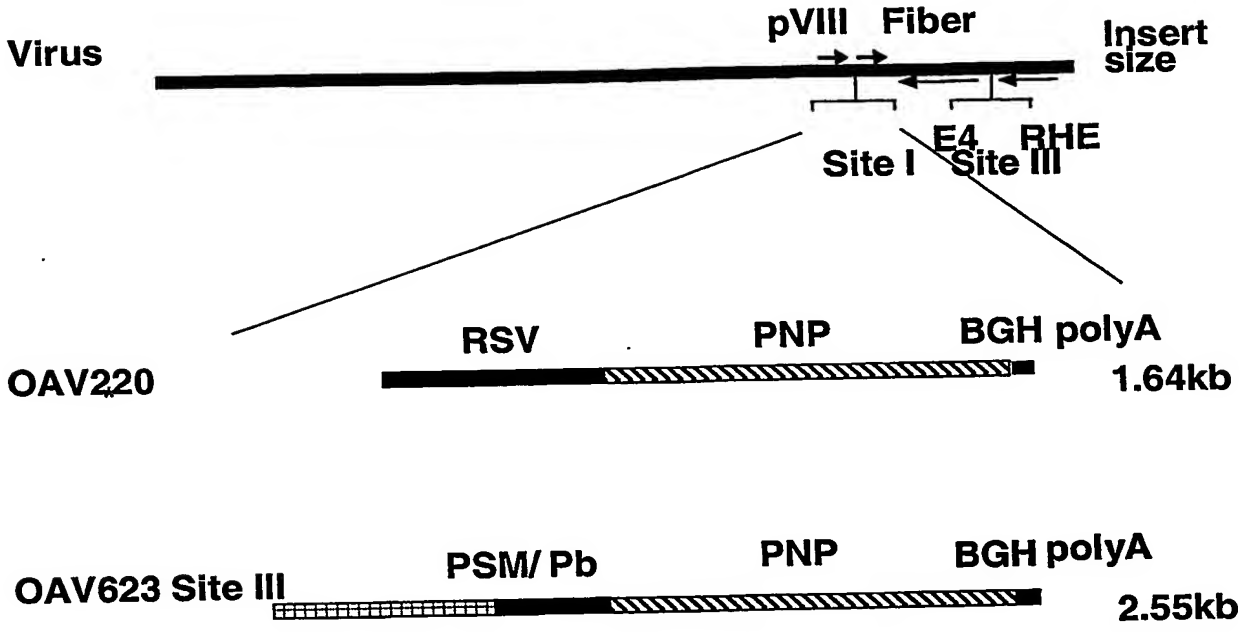


Figure 1. Key to recombinant adenoviruses used in the GDEPT studies described in this specification and the gene expression cassettes they carry. **OAdV220** expresses PNP from the constitutive RSV promoter (Long terminal repeat from the Rous Sarcoma Virus). The insertion is in site 1 in the OAdV genome. **OAdV222** is the same as OAdV 220 with the exception that the RSV promoter is replaced with the constitutive human cytomegalovirus immediate early promoter. **OAdV623** expresses PNP from the chimaeric PSM/Pb promoter. The PSM enhancer is depicted in vertical lines, the probasin promoter is solid. This promoter drives prostate-specific expression of PNP. This expression cassette is inserted into site III in the OAdV genome depicted at the top of the figure. **OAdV223** carries the same expression cassette as OAdV623 but inserted into site I of the OAdV genome. All expression cassettes carry the transcription termination sequences from the bovine growth hormone gene (BGH polyA).

Figure 2 Alkaline phosphatase staining at day four post infection in human PC3 prostate tumours. Tumours were injected with OAdV216 ($1.2x10^8$pfu). OAdV216 expresses the human placental alkaline phosphatase reporter gene. Black regions represent zones of alkaline phosphatase expression. Magnification 5X

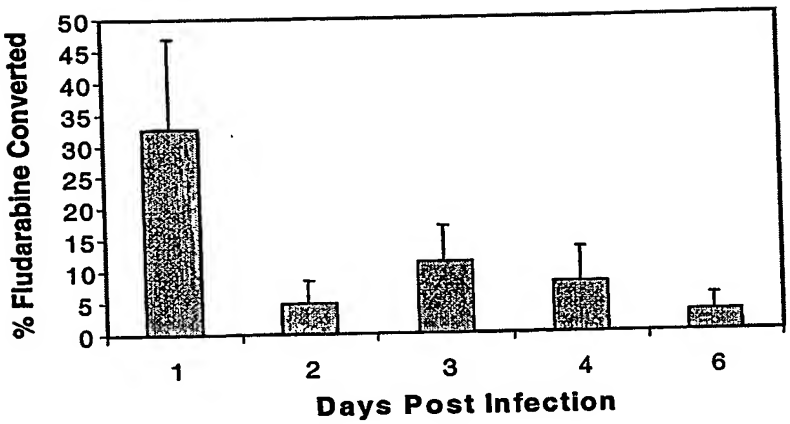


Figure 3. In vitro conversion of Fludarabine to 2FA by PNP activity in RM1 tumour extracts. Tumours were inoculated with OAdV220 at 6 x 10^9 VP per tumour. Tumours were harvested 1-6 days after treatment and homogenised. Homogenates (500 μg of protein) were incubated with 500 n moles of Fludarabine for 16h at 37°C. % Conversion of Fludarabine to 2FA was determined by HPLC analysis.

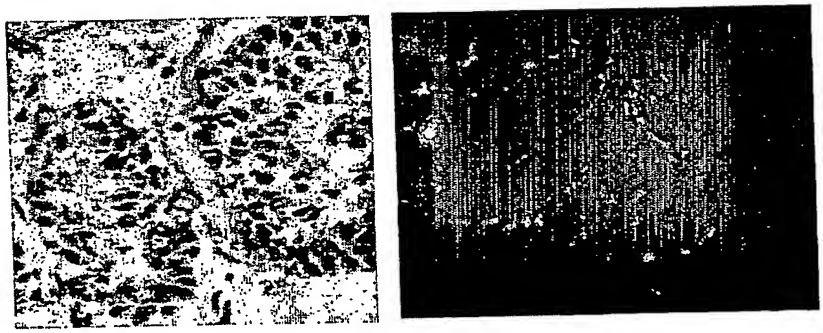


Figure 4. Transduction of TURP tissue with OAdVGFP. Post-surgery human prostate tissue was infected with an OAdV vector carrying the GFP reporter gene. Left panel, Histological staining of a section through a. Right panel, green fluorescence at sites susceptible to virus infection seen in a whole cultured TURP fragment transduced with OAdV217A.

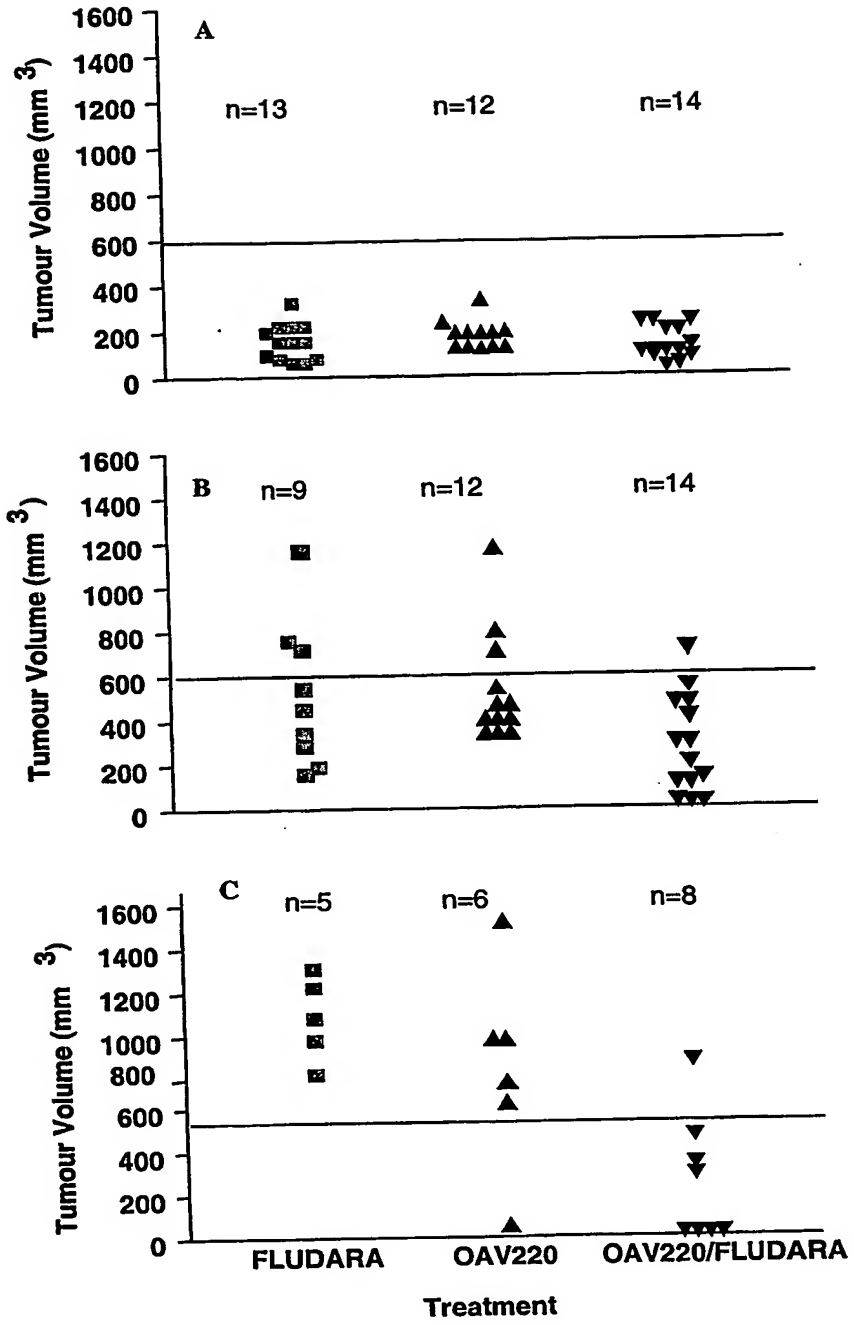


Figure 5. Effect of OAdV and FLUDARA treatment on sc PC-3 tumour growth in nude mice. Animals were weighed, and tumour diameters were measured twice weekly. Data show the spread of tumour sizes and the number of survivors at days A, 4; B, 25 and C, 53 post-tumour injection for treatment and control groups. The line indicates half the maximum volume that tumours were allowed to grow to. Note that four tumours in the OAdV220/Fludarabine treatment group had completely regressed by day 53.

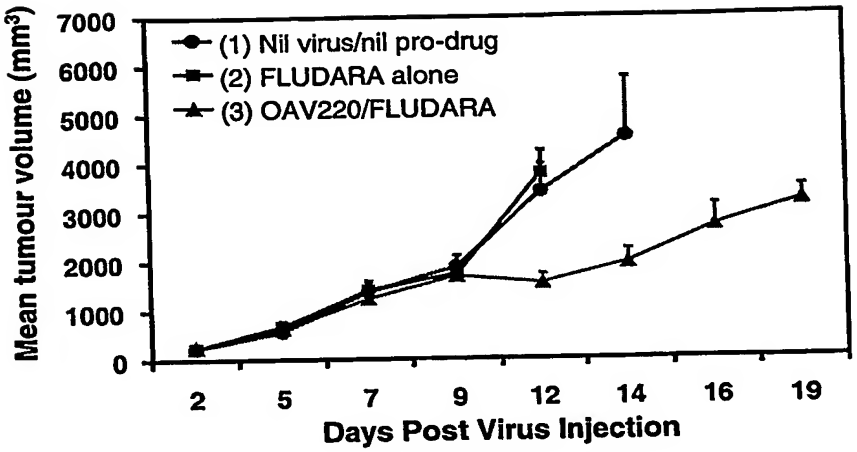


Figure 6. Effect of fludarabine and OAdV220 transduction on the growth of RM-1 tumours.

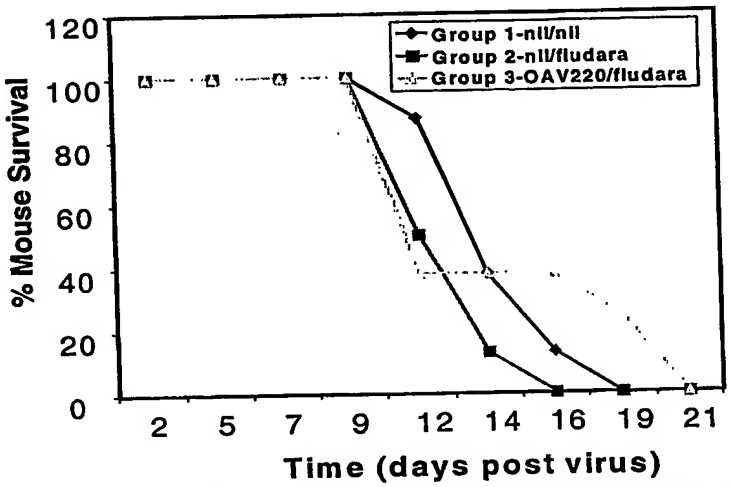


Figure 7. Effect of fludarabine and OAdV 220 on survival of animals bearing RM-1 subcutaneous tumours.

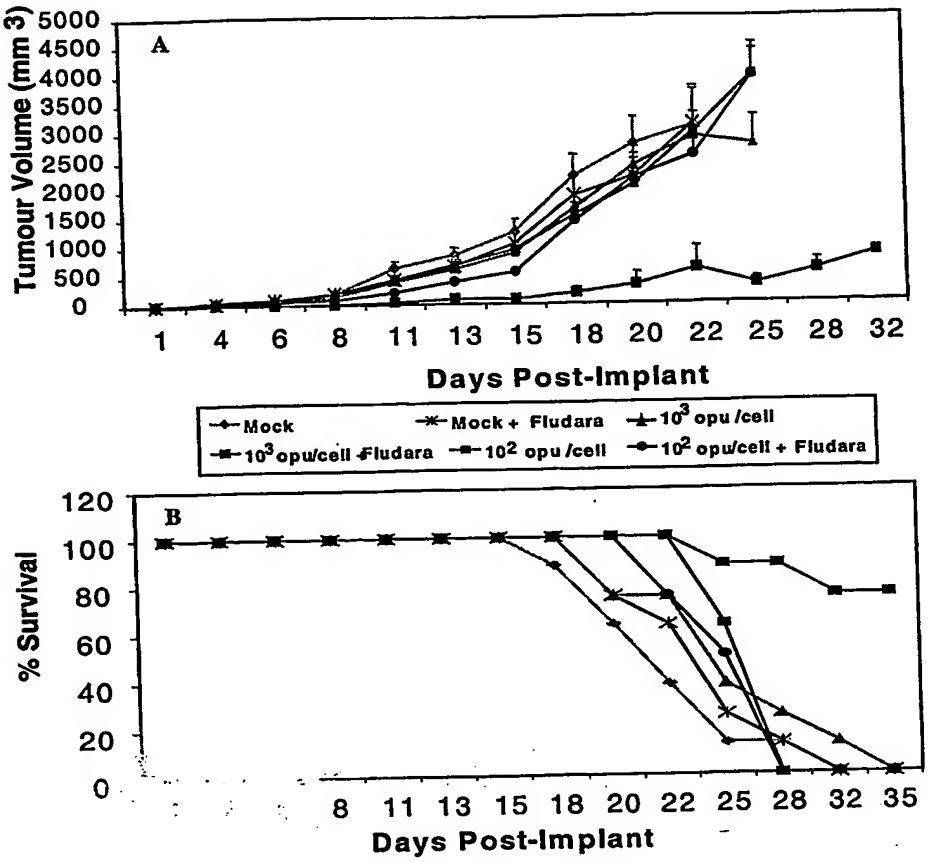
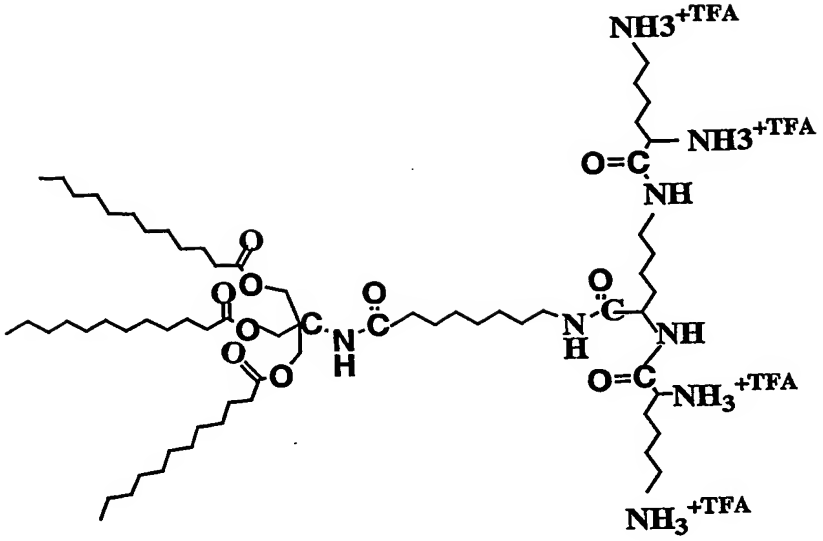
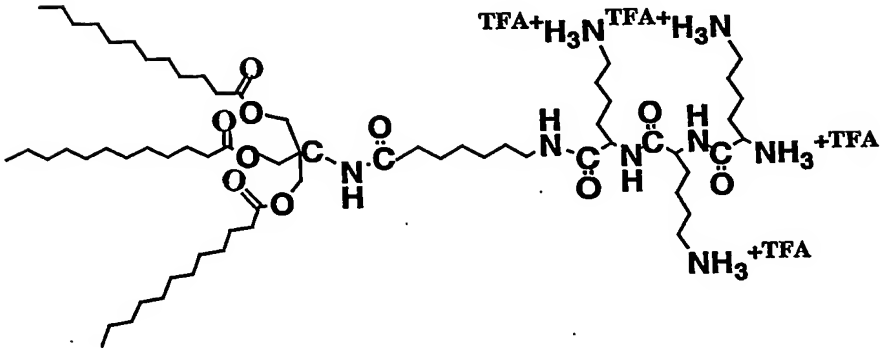


Figure 8. Effect of pre-transduction of RM-1 cells with OAdV220 on GDEPT in subcutaneous tumours. RM-1 cells were pre-treated with OAdV220 or virus storage buffer as indicated in the key on day −1. Mice received daily ip injections of either fludarabine or saline. A, Impact of GDEPT on tumour volume (mean + SE). B, Impact of GDEPT on mouse survival.



CSO87



CS060

Figure 9. Examples of Tris conjugate cationic lipids.

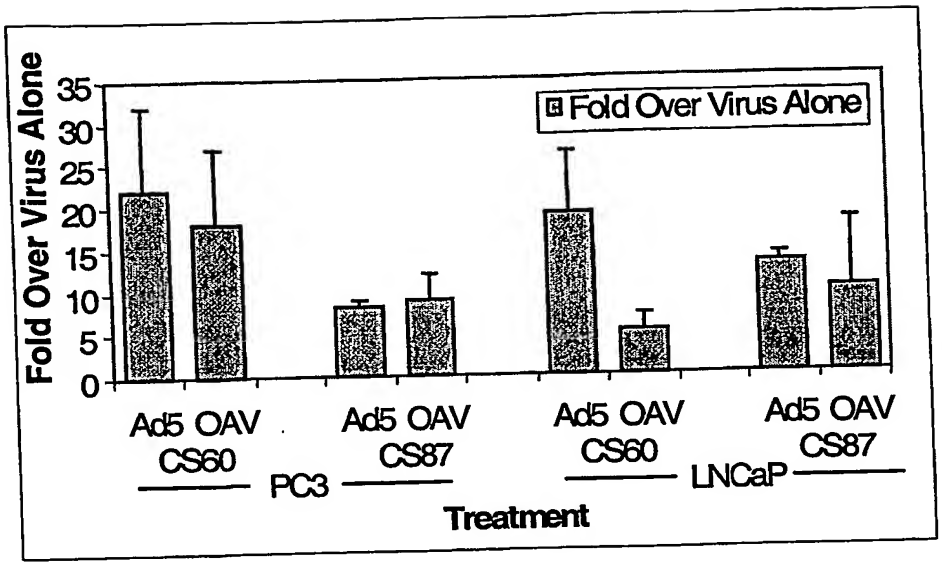


Figure 10. Effect of Tris-conjugated cationic lipids on vector transduction in prostate cancer cells in vitro. The Ad5 and OAdV vectors carried reporter gene cassettes encoding E. coli β-galactosidase and human placental alkaline phosphatase respectively. Levels of expression obtained in the presence of the lipids indicated (CS60 and CS87) are expressed relative to those achieved with the same virus in the absence of lipid. Transduction experiments were performed in vitro in two human prostate cancer cell lines, PC3 and LNCaP. Bars indicate Standard Errors.

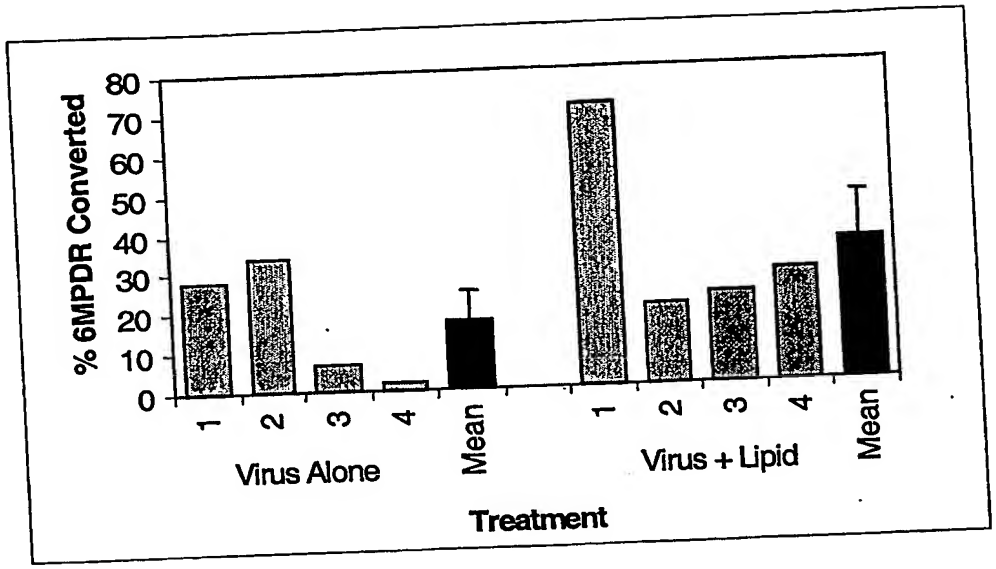


Figure 11. Lipid enhancement of OAdV transduction *in vivo*. Human LN3 prostate cancer tumours were grown subcutaneously in nude mice. Tumours were injected with 10^{10} [illegible] dV623 (a vector carrying the *E. coli* purine nucleoside phosphorylase [illegible] under the control of the PSM/Pb promoter) in the presence or [illegible] cationic lipid CS87. Tumours were excised 3 days later, homogenised and efficiency of transduction was determined by an assay for PNP activity. Tumour lysate protein (500µg) was incubated with the PNP substrate 6MPDR for 2h at 37°C and the amount of conversion to 6MP was determined by HPLC analysis. Absolute values for each tumour plus mean values and standard errors for the different treatments are shown.

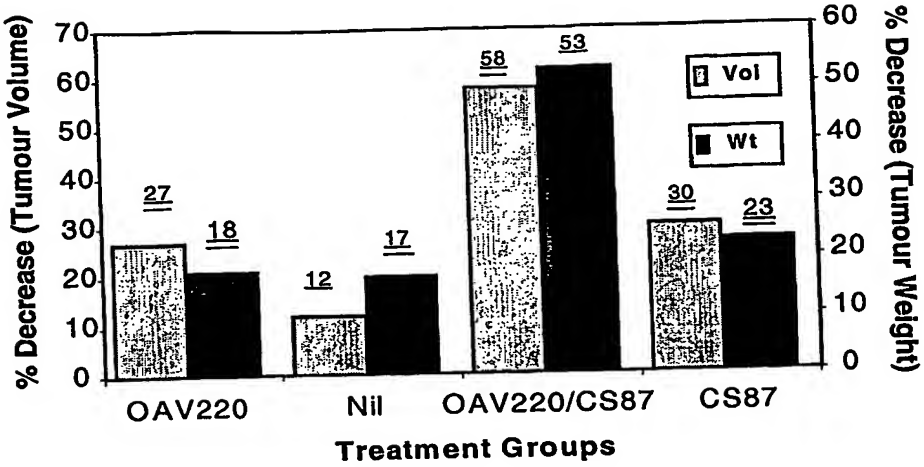


Figure 12. Effect of GDEPT ± lipid on growth of intraprostatic RM-1 tumours. RM-1 prostate tumours were established orthotopically and treated as indicated on the X-axis. Tumours were removed on day 18 and their volumes and weights determined. Data represent the reduction in tumour volume or weight observed for any given treatment in the presence of Fludarabine [illegible] at of the equivalent non-Fludarabine treated tumour expressed a[illegible] olume or weight of the non-prodrug treated tumour.

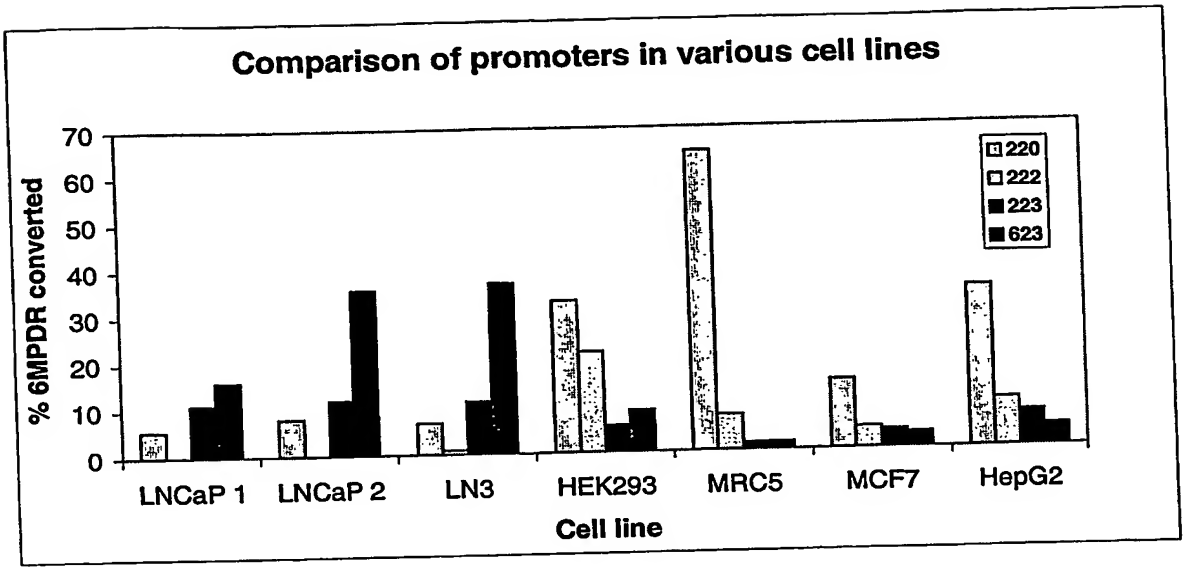


Figure 14. Comparison of PSM/Pb and non-specific promoter activities during OAdV infections in various cell types. PNP production was measured by 6MPDR conversion. As not all cell types were equally infected by OAdV and different amounts of cell lysate were assayed, only the ratio of activities within a cell type should be compared. These human cell lines were derived from breast (MCF7) or liver (HepG2) or prostate (LNCaP, LN3) cancers. MRC5 cells are normal human lung fibroblasts.

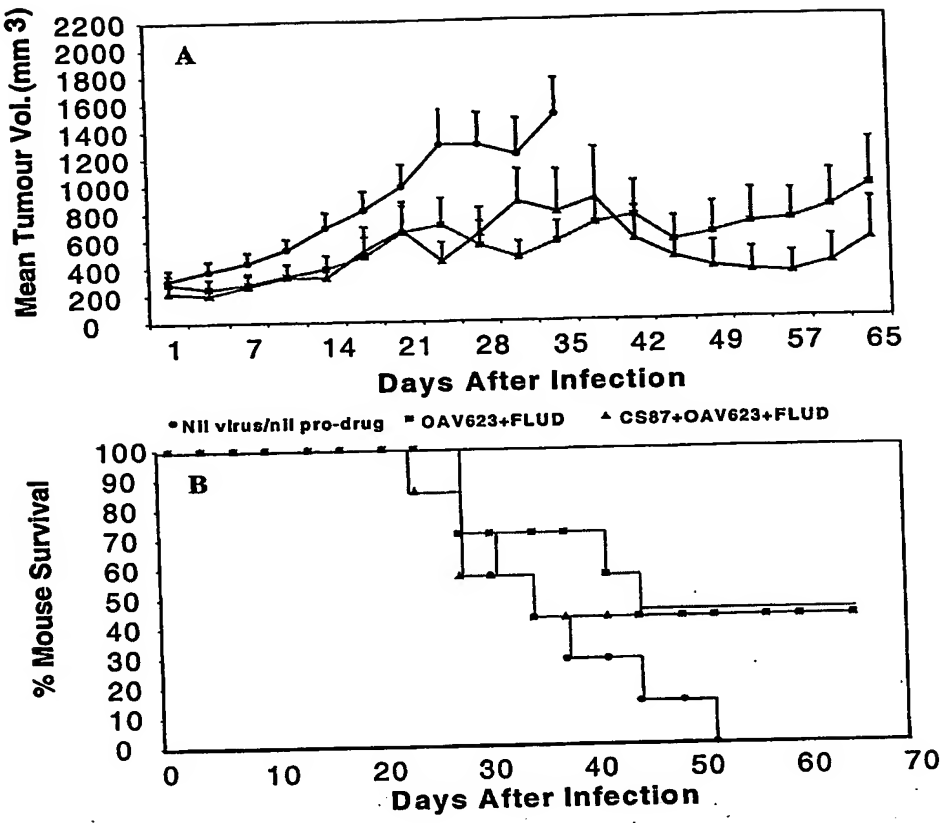


Figure 15. Effect of prostate specific expression of the GDEPT on growth of human prostate tumours in vivo and on host survival (nude mice). Tumours were grown subcutaneously in nude mice from human LN3 prostate cancer cells. Tumours were injected with OAdV623 carrying the PNP gene under the control of the prostate specific PSM/Pb promoter in the presence or absence of cationic lipid (CS87). Test animals received Fludarabine for 5 days post virus injection. A; effect on tumour growth, B, effect on mouse survival. Treatment groups are indicated in the key.

CLAIMS

1. A method of treating a solid tumour of a specific tissue type in a subject, the method comprising the following steps

(i) delivering to the solid tumour a composition comprising an engineered ovine adenovirus; and

(ii) administering a prodrug to the subject,

wherein the engineered ovine adenovirus comprises a promoter active in the specific tissue and a gene encoding an enzyme which converts the prodrug to a cytotoxic metabolite, the gene being under the control of the tissue specific promoter.

2. A method as claimed in claim 1 in which the specific tissue type is prostate tissue.

3. A method as claimed in claim 1 or claim 2 in which the tissue specific promoter is the prostate specific membrane antigen promoter or the probasin promoter.

4. A method as claimed in any one of claims 1 to 3 in which the ovine adenovirus further comprises a transcri[illegible]er element, preferably the enhancer element from the prostate [illegible]brane antigen gene.

5. A method as claimed in any one of claims 1 to 4 in which the enzyme is a purine nucleoside phosphorylase (PNP), and the prodrug is a purine pro-drug which is converted by PNP to a toxic purine metabolite.

6. A method as claimed in claim 5 in which the prodrugs is 6-methyl purine-2-deoxyriboside (6MPDR) or fludarabine.

7. A method as claimed in any one of claims 1 to 6 in which the composition comprising the engineered ovine adenovirus further comprises a lipid. It is presently preferred that the lipid is a cationic lipid.

8. A method as claimed in any one of claims 1 to 7 in which the composition comprising the engineered ovine adenovirus is delivered directly to the solid tumour by injecting the composition into the tumour.